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Molecular evolution of plant AAP and LHT amino acid transporters

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Nitrogen is an essential mineral nutrient and it is often transported within living organisms in its reduced form, as amino acids. Transport of amino acids across cellular membranes requires proteins, and here we report the phylogenetic analysis across taxa of two amino acid transporter families, the amino acid permeases (AAPs) and the lysine–histidine-like transporters (LHTs). We found that the two transporter families form two distinct groups in plants supporting the concept that both are essential. AAP transporters seem to be restricted to land plants. They were found in *Selaginella moellendorffii* and *Physcomitrella patens* but not in Chlorophyte, Charophyte, or Rhodophyte algae. AAPs were strongly represented in vascular plants, consistent with their major function in phloem (vascular tissue) loading of amino acids for sink nitrogen supply. LHTs on the other hand appeared prior to land plants. LHTs were not found in chlorophyte algae *Chlamydomonas reinhardtii* and *Volvox carterii*. However, the characean alga *Klebsormidium flaccidum* encodes KfLHT13 and phylogenetic analysis indicates that it is basal to land plant LHTs. This is consistent with the hypothesis that characean algae are ancestral to land plants. LHTs were also found in both *S. moellendorffii* and *P. patens* as well as in monocots and eudicots. To date, AAPs and LHTs have mainly been characterized in *Arabidopsis* (eudicots) and these studies provide clues to the functions of the newly identified homologs.

Keywords: amino acid, nitrogen, transporter, AAP, LHT, membrane, evolution

INTRODUCTION

Nitrogen is a critical mineral nutrient in all living organisms since it is required for synthesis of a large number of compounds including hormones, nucleotides, and amino acids. As the basic building blocks of proteins, amino acids are needed for metabolism, cellular structure, growth, and development. Amino acid uptake into cells and cellular compartments depends on membrane-integral transporter proteins, and amino acid transporters have been identified in many organisms including bacteria, fungi, animals, and plants (Chang et al., 2004; Boudko, 2010). In plants, amino acid transporters are found in two families within the amino acid–polyamine–choline (APC) transporter superfamily, the amino acid/auxin permease (AAAP), and the APC family. The AAAP family includes transporters from plants, animals, and fungi (Chang et al., 2004), and in plants contains the amino acid permeases (AAPs), lysine–histidine-like transporters (LHTs), proline transporters (ProTs), γ-aminobutyric acid transporters (GATs), ANT1-like aromatic, and neutral amino acid transporters and auxin transporters (AUXs; Wipf et al., 2002; Rentsch et al., 2007). Cationic amino acid transporters (CATs) belong to the APC family and are present in both animals and plants[1])

This study addresses the phylogeny of the plant AAP and LHT transporters. These have been characterized in angiosperms (flowering plants), and specifically in eudicots, and detailed overviews on their substrate specificity, localization, and biological functions have recently been presented (see Fischer et al., 2002; Lee and Tegeder, 2004; Rentsch et al., 2007; Tegeder and Rentsch, 2010; Tegeder et al., 2011). In *Arabidopsis* the AAP family consists of eight members (AtAAP1–8) that generally transport neutral and acidic amino acids with moderate affinity, with the exception of AtAAP3 and AtAAP5 that also transport basic amino acids (Fischer et al., 1995, 2002; Rentsch et al., 2007; Svennerstam et al., 2008). All *Arabidopsis* AtAAPs analyzed to date have been localized to the plasma membrane and they function as H^+-coupled amino acid uptake systems (see Tegeder and Rentsch, 2010). AAPs have been suggested to be involved in a number of physiological processes in plants including amino acid uptake from the soil (Hirner et al., 2006; Lee et al., 2007; Svennerstam et al., 2008), phloem loading or xylem–phloem transfer (Schulze et al., 1999; Okumoto et al., 2002; Koch et al., 2003; Tegeder et al., 2007; Tan et al., 2008; Hunt et al., 2010; Zhang et al., 2010; see also Tegeder and Rentsch, 2010), and seed loading (Schmidt et al., 2007; Tegeder et al., 2007; Tan et al., 2008; Sanders et al., 2009).

Much less is known about the LHTs, a family of 10 members (AtLHT1–10) in *Arabidopsis*. AtLHT1 was originally described as a lysine and histidine selective transporter (Chen and Bush, 1997), but other studies with AtLHT1 and AtLHT2 suggests that LHTs preferentially transport neutral and acidic amino acids with high affinity (Lee and Tegeder, 2004; Hirner et al., 2006; Svennerstam et al., 2007, 2008). Like the AAPs, AtLHTs are localized to the plasma membrane and transport a broad spectrum of amino acids

[1]http://www.tcdb.org/superfamily.php

from the cell wall space into the cell (Hirner et al., 2006; Foster et al., 2008). Based on promoter–*GUS* studies, LHTs have been suggested to be involved in import of organic nitrogen into root and mesophyll cells (Hirner et al., 2006), as well as into pollen and other cells of reproductive floral tissue (Lee and Tegeder, 2004; Foster et al., 2008).

AAPs and LHTs have not yet been described in any organisms other than angiosperms. With the recent progress in genome sequencing we are however now in the excellent position to determine whether AAP and LHT amino acid transporters are present in ancestors of seed plants and to examine the phylogenetic relationship of AAP and LHT proteins. Three major clades form the large monophyletic plant kingdom. These include the green plants (Viridiplantae), Rhodophytes (red algae), and Glaucophytes (freshwater microscopic algae; **Figure 1**; Anderberg et al., 2011). The green plants are grouped into the Chlorophytes that contain algae such as *Chlamydomonas reinhardtii*, and the Streptophytes with algal species (Charophytes) and land plants (Finet et al., 2010; Banks et al., 2011). The land plants are divided in non-vascular plants (Bryophytes; i.e., liverworts, mosses, and hornworts) and vascular plants that split into Lycophytes (non-seed plants) and Euphyllophytes. The Lycophytes contain clubmosses, quilworts, and spikemosses while Euphyllophytes consist of ferns (Monilophytes) and seed-bearing plants (Spermatophytes), which are often grouped into angiosperms (flowering plants) and gymnosperms (i.e., cycads, *Ginkgo*, conifers, and gnetophytes). However, the evolutionary relationships of Spermatophytes are not clearly resolved (Magallon and Sanderson, 2002; Mathews, 2009).

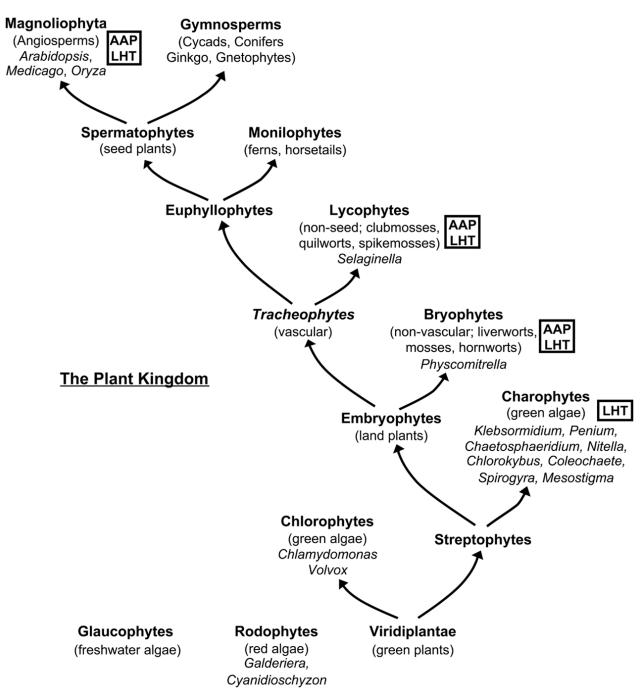

FIGURE 1 | Simplified overview of the plant kingdom and analyzed genera. Taxa containing AAPs and/or LHTs are marked. Note, that genomes of gymnosperms and monilophytes have not been used for the analysis.

Here, sequences from red algae (*Galdieria sulfuraria* and *Cyanidioschyzon merolae*), green algae (Chlorophytes: *Chlamydomonas reinhardtii* and *Volvox carterii*; Charophytes: *Penium marinum*, *Spirogyra praetensis*, *Coleochaete* sp., *Chaetosphaeridium globosum*, *Mesostigma viride*, *Nitella hyalina*, *Klebsormidium flaccidum*, *Chlorokybus atmosphyticus*), and basal non-vascular (*Physcomitrella patens*), non-seed vascular (*Selaginella moellendorffii*), and vascular land plants (eudicots: *Arabidopsis thaliana*, *Medicago sativa*; monocots: *Oryza sativa*) were analyzed for AAP and LHT proteins (**Figure 1**). Phylogenetic reconstruction was performed to determine diversification of the AAP and LHT amino acid transporters as well as their lineage association.

RESULTS AND DISCUSSION

AAP AND LHT TRANSPORTERS FORM TWO DISTINCT GROUPS

Database searches for AAP and LHT proteins in red algae, green algae, basal non-vascular and vascular land plants, and seed plants resulted in 44 AAP and 39 LHT protein sequences (**Figure 2**; **Tables 1** and **2**). Predicted protein sequences for the AAPs averaged 478 ± 14 amino acids (mean ± SD). The length of LHT sequences was similar (463 ± 31 amino acids). The LHT sequence from *Klebsormidium flaccidum* (KfLHT13) is an incomplete cDNA and contains the C-terminal 388 amino acids. A maximum-likelihood

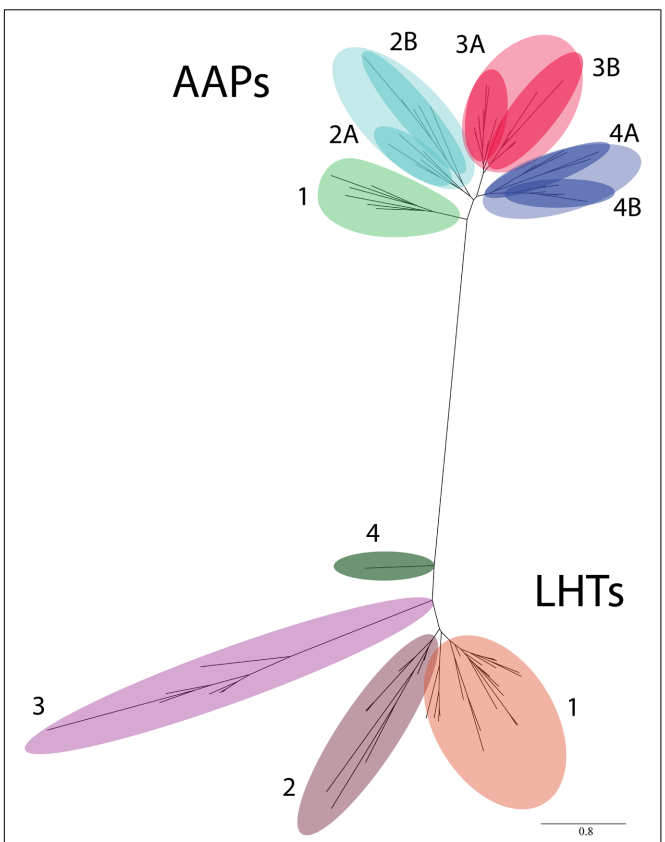

FIGURE 2 | Phylogenetic tree of AAP and LHT proteins found across taxa. A total of 44 AAP and 39 LHT proteins were identified. AAPs and LHTs form two distinct groups, each sub-divided in clusters or subclusters. The clusters (numbers) and subclusters (letters) were labeled and color-coded.

Table 1 | Amino acid permeases protein sequences sorted by subcluster.

Abbreviation	Gene	Organism	Protein size (aa)
SUBCLUSTER 1			
AtAAP7	At5g23810	*Arabidopsis thaliana*	467
MtAAP7A	Medtr3g080570	*Medicago truncatula*	460
MtAAP7B	Medtr5g104490	*Medicago truncatula*	462
OsAAP7A	Os04g39489	*Oryza sativa*	466
OsAAP7B	Os04g56470	*Oryza sativa*	469
OsAAP7C	Os02g49060	*Oryza sativa*	469
SUBCLUSTER 2A			
PpAAP9A	Pp1s107_87V6[1]	*Physcomitrella patens*	515
PpAAP9B	Pp1s387_41V6[1]	*Physcomitrella patens*	500
SmAAP9A	442676[1]	*Selaginella moellendorffii*	503
SmAAP9B	166966[1]	*Selaginella moellendorffii*	467
SmAAP9C	90661[1]	*Selaginella moellendorffii*	479
SUBCLUSTER 2B			
OsAAP10A	Os06g12350	*Oryza sativa*	507
OsAAP10B	Os12g09300	*Oryza sativa*	468
OsAAP10C	Os01g65660	*Oryza sativa*	465
OsAAP10D	Os01g65670	*Oryza sativa*	466
SmAAP10	442677[1]	*Selaginella moellendorffii*	495
SUBCLUSTER 3A			
AtAAP2	At5g09220	*Arabidopsis thaliana*	493
AtAAP3	At1g77380	*Arabidopsis thaliana*	476
AtAAP4	At5g63850	*Arabidopsis thaliana*	466
AtAAP5	At1g44100	*Arabidopsis thaliana*	480
MtAAP2A	Medtr4g143430	*Medicago truncatula*	475
MtAAP2B	Medtr5g017170	*Medicago truncatula*	465
MtAAP2C	Medtr3g142750	*Medicago truncatula*	466
MtAAP2D	Medtr3g142780	*Medicago truncatula*	477
MtAAP2E	Medtr3g142720	*Medicago truncatula*	465
OsAAP3	Os02g01210	*Oryza sativa*	518
SUBCLUSTER 3B			
OsAAP11A	Os12g08090	*Oryza sativa*	475
OsAAP11B	Os12g08130	*Oryza sativa*	475
OsAAP11C	Os11g09020	*Oryza sativa*	476
OsAAP11D	Os12g09320	*Oryza sativa*	468
OsAAP11E	Os01g66010	*Oryza sativa*	488
OsAAP11F	Os05g34980	*Oryza sativa*	496
OsAAP11G	Os04g41350	*Oryza sativa*	471
SUBCLUSTER 4A			
OsAAP12A	Os06g36180	*Oryza sativa*	487
OsAAP12B	Os06g36210	*Oryza sativa*	474
OsAAP12C	Os06g12330	*Oryza sativa*	484
MtAAP12A	Medtr1g008290	*Medicago truncatula*	457
MtAAP12B	Medtr1g008320	*Medicago truncatula*	473
SUBCLUSTER 4B			
AtAAP1	At1g58360	*Arabidopsis thaliana*	485
AtAAP6	At5g49630	*Arabidopsis thaliana*	481
AtAAP8	At1g10010	*Arabidopsis thaliana*	475
MtAAP6A	Medtr1g008410	*Medicago truncatula*	481
MtAAP6B	Medtr3g127950	*Medicago truncatula*	491
OsAAP6	Os07g04180	*Oryza sativa*	487

[1] *Phytozome gene identifier.*

Table 2 | Lysine–histidine-like transporters protein sequences sorted by subcluster.

Abbreviation	Gene	Organism	Protein size (aa)[1]
SUBCLUSTER 1			
AtLHT1	At5G40780	*Arabidopsis thaliana*	446
AtLHT2	At1G24400	*Arabidopsis thaliana*	441
AtLHT3	At1G61270	*Arabidopsis thaliana*	451
AtLHT5	At1G67640	*Arabidopsis thaliana*	441
AtLHT6	At3G01760	*Arabidopsis thaliana*	455
AtLHT8	At1G71680	*Arabidopsis thaliana*	448
AtLHT9	At1G25530	*Arabidopsis thaliana*	440
AtLHT10	At1G48640	*Arabidopsis thaliana*	453
MtLHT1A	Medtr2g122930	*Medicago truncatula*	453
MtLHT1B	Medtr6g025000	*Medicago truncatula*	484
MtLHT2A	AC233656_24.1	*Medicago truncatula*	471
MtLHT2B	Medtr3g103290	*Medicago truncatula*	436
MtLHT3	Medtr8g109640	*Medicago truncatula*	425
MtLHT8	Medtr3g013200	*Medicago truncatula*	469
MtLHT9A	Medtr1g117410	*Medicago truncatula*	437
MtLHT9B	Medtr1g117800	*Medicago truncatula*	437
MtLHT9C	Medtr1g117420	*Medicago truncatula*	437
MtLHT9D	Medtr1g117790	*Medicago truncatula*	437
OsLHT1	Os08g03350	*Oryza sativa*	447
OsLHT2	Os12g14100	*Oryza sativa*	446
OsLHT8	Os05g14820	*Oryza sativa*	456
OsLHT9	Os04g38860	*Oryza sativa*	444
PpLHT11A	Pp1s79_71V6.1[2]	*Physcomitrella patens*	480
PpLHT11B	Pp1s105_62V6.1[2]	*Physcomitrella patens*	465
PpLHT11C	Pp1s5_176V6.1[2]	*Physcomitrella patens*	453
SmLHT11A	270979[2]	*Selaginella moellendorffii*	473
SmLHT11B	127260[2]	*Selaginella moellendorffii*	430
SUBCLUSTER 2			
PpLHT11A	Pp1s79_71V6.1[2]	*Physcomitrella patens*	480
PpLHT11B	Pp1s105_62V6.1[2]	*Physcomitrella patens*	465
PpLHT11C	Pp1s5_176V6.1[2]	*Physcomitrella patens*	453
SmLHT11A	270979[2]	*Selaginella moellendorffii*	473
SmLHT11B	127260[2]	*Selaginella moellendorffii*	430
SmLHT11C	75458[2]	*Selaginella moellendorffii*	427
SmLHT11D	17345[2]4	*Selaginella moellendorffii*	468
SmLHT11E	12727[2]0	*Selaginella moellendorffii*	450
SUBCLUSTER 3			
AtLHT4	At1G47670	*Arabidopsis thaliana*	519
AtLHT7	At4G35180	*Arabidopsis thaliana*	478
MtLHT4A	Medtr2g014200	*Medicago truncatula*	520
MtLHT4B	Medtr2g013940	*Medicago truncatula*	520
MtLHT7	Medtr5g023220	*Medicago truncatula*	534
OsLHT4A	Os12g30040	*Oryza sativa*	508
OsLHT7	Os04g47420	*Oryza sativa*	512
PpLHT4	Pp1s77_57V6.1[2]	*Physcomitrella patens*	559
SUBCLUSTER 4			
KfLHT13	kfla_Contig1880	*Klebsormidium flaccidum*	(388)

[1] *Partial sequences are listed in parentheses.*

[2] *Phytozome gene identifier.*

tree was constructed using PhyML 3.0 (Guindon et al., 2010) based on the alignment of full-length AAP and LHT sequences and the truncated KfLHT13 (**Figure 2**). In addition, trees were made using alignments in which the variable-length N- and C-terminal

regions of the alignment were removed (data not shown). These trees did not differ from those based on full-length AAPs and LHTs, and KfLHT13 (**Figures 2–4**). Both AAPs and LHTs were found in eudicots, monocots, *Selaginella* and *Physcomitrella*, but AAPs and LHTs form two distinct groups supporting functional differences between the two transporter families in the analyzed organisms (see also **Figure 1**). The absence of AAP or LHT genes in Chlorophytes is consistent with the hypothesis that chlorophyte algae are not ancestors of land plants (Turmel et al., 1999; Karol et al., 2001; Kapraun, 2007).

AAPs EVOLVED AT THE SAME TIME AS LAND PLANTS

When searching the databases, AAP proteins were found in non-vascular land plants (*Physcomitrella patens*; 2 proteins), non-seed vascular plants (*Selaginella moellendorffii*, 4 proteins), and seed plants including *Arabidopsis thaliana* (8 proteins), *Oryza sativa* (19 proteins), and *Medicago truncatula* (11 proteins). No AAPs were found in algal sequences of Rhodophytes (*Galdieria* and *Cyanidioschyzon*), Chlorophytes (*Chlamydomonas* and *Volvox*), or Charophytes (*Penium*, *Spirogyra*, *Coleochaete*, *Chaetosphaeridium*, *Nitella*, *Klebsormidium*, and *Chlorokybus*). However, the Charophyte search was based on EST sequences, and until the whole genome sequences are available we cannot rule out that AAPs are present in Charophytes. The identified AAP proteins are grouped into four main clusters (1, 2, 3, and 4), with cluster 2–4 being subdivided into two subclusters A and B (**Table 1**; **Figure 3**). Cluster 1, 3, and 4 only contain AAPs of seed plants while cluster 2 contains non-vascular and non-seed vascular plant, and angiosperm proteins.

Cluster 1 contains proteins from monocots and eudicots that are related to *Arabidopsis* AtAAP7. It holds AtAAP7 and two *Medicago* proteins (MtAAP7A and 7B) consistent with a genome duplication in legumes relative to *Arabidopsis* (Cannon et al., 2006). In addition, it includes three rice AAPs (OsAAP7A–7C) that likely represent an amplification of AAP7 genes in monocots. While the specific function of AtAAP7 and related proteins is still unknown, the phylogenetic analysis supports that they are important for seed plants since they are maintained in both monocot and eudicot lineages.

Cluster 2 contains AAP proteins from non-vascular and non-seed plants, and monocots, but lacks eudicot proteins. Subcluster 2A includes proteins only from the moss *Physcomitrella patens* (PpAAP9A and 9B) and spikemoss *Selaginella moellendorffii* (SmAAP9A–9C), but no proteins from seed plants, suggesting differences in amino acid transporter function between early and higher land plants. Differences in function might be based on (i) differences in phloem loading or source-sink transport between Spermatophytes that have complex leaf venation and the Bryophytes and Lycophytes with no vasculature or microphylls with only a single vascular strands (Reinhart and Thomas, 1981; Aldous, 2002; Beerling, 2005), or on (ii) differences in reproduction (flower versus spores and spore-bearing structures; Prigge and Bezanilla, 2010). For example, specific transporters might be needed for uptake of amino acids into moss sporophytes (Caussin et al., 1983).

In contrast, subcluster 2B contains four proteins from rice (OsAAP10A–10D) and one from *S. moellendorffii*. It is interesting

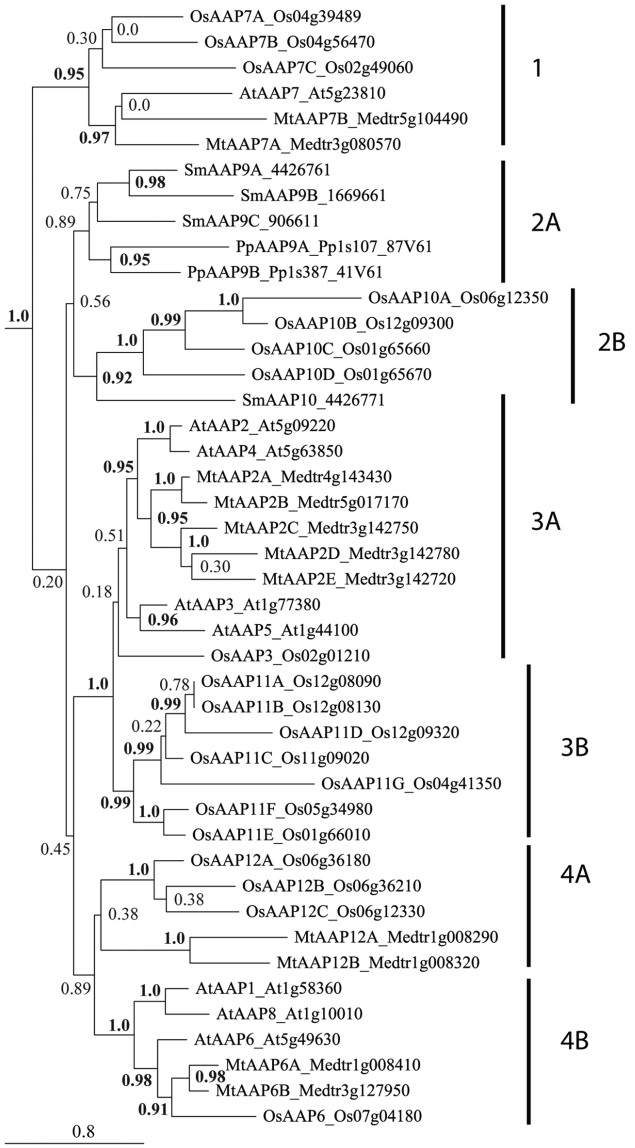

FIGURE 3 | Phylogenetic analysis of AAP proteins found in *Arabidopsis thaliana* (At), *Oryza sativa* (Os), *Medicago truncatula* (Mt), *Selaginella moellendorffii* (Sm), and *Physcomitrella patens* (Pp). Multiple protein sequence alignment was done using CLUSTAL X (Thompson et al., 2007). A maximum-likelihood tree was constructed using PhyML 3.0 (Guindon et al., 2010). Numbers at the nodes indicate SH-like branch support (Shimodaira and Hasegawa, 1999; Buckley et al., 2001). Values above 0.9 (in bold) show significant phylogenetic support. Accession numbers of sequences (see **Table 1**) are provided after the species transporter names. The clusters (numbers) and subclusters (letters) are labeled.

that cluster 2 lacks eudicot representation, which indicates that AAPs of this type were lost from eudicot genomes after divergence from monocots. This also suggests that AAPs in cluster 2 represent the earliest AAP sequences. No information is available concerning the function of AAPs in cluster 2. However, the presence of monocot AAPs indicates that an essential amino acid transport activity, required for non-vascular and non-seed vascular plants,

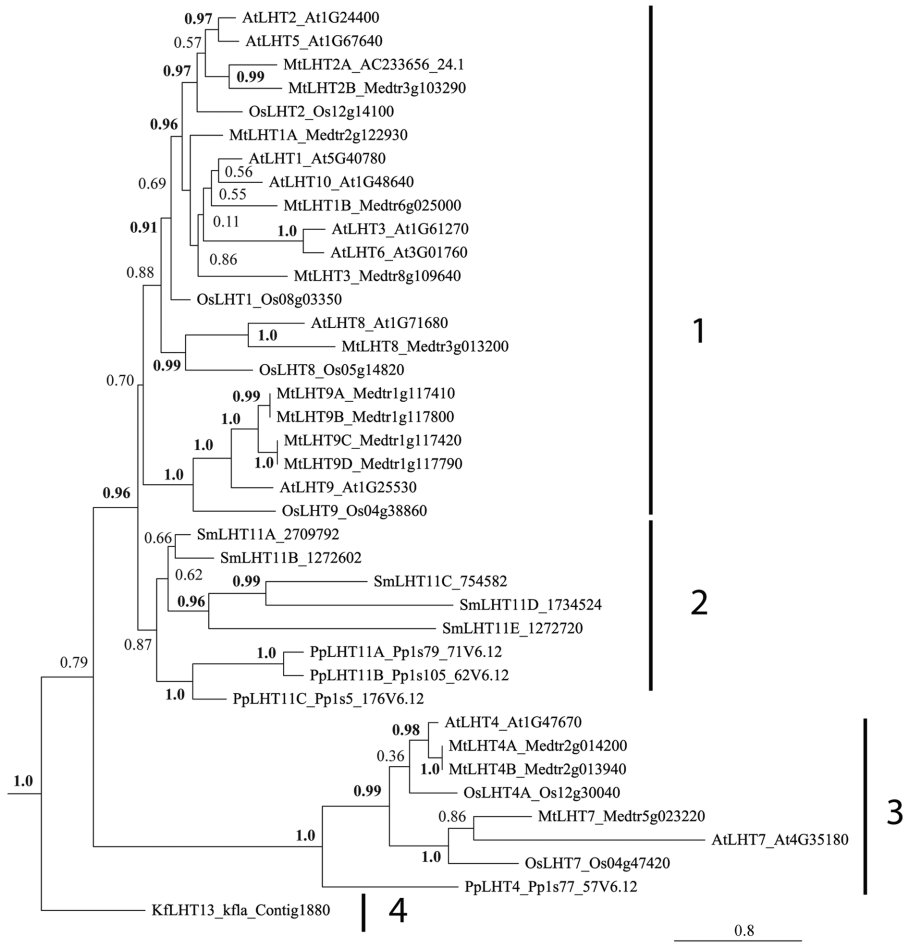

FIGURE 4 | Phylogenetic analysis of LHT proteins found in *Arabidopsis thaliana* (At), *Oryza sativa* (Os), *Medicago truncatula* (Mt), *Selaginella moellendorffii* (Sm), *Physcomitrella patens* (Pp), and *Klebsormidium flaccidum* (Kf). Multiple protein sequence alignment was done using CLUSTAL X (Thompson et al., 2007). A maximum-likelihood tree was constructed using PhyML 3.0 (Guindon et al., 2010). Numbers at the nodes indicate SH-like branch support (Shimodaira and Hasegawa, 1999; Buckley et al., 2001). Values above 0.9 (in bold) show significant phylogenetic support. Accession numbers of sequences (see **Table 2**) are provided after the species transporter names. The clusters are numbered.

was maintained in monocots and likely replaced in eudicots by other AAPs or amino acid transporters in other families.

Cluster 3 contains only monocot and eudicot sequences. Subcluster 3A includes *Arabidopsis* AtAAP3 and AtAAP5 and one related rice protein (OsAAP3), as well as *Arabidopsis* AtAAP2 and AtAAP4 and five legume/*Medicago* AAPs (MtAAP2A–2E). All of the *Arabidopsis* AtAAPs in subcluster 3A appear to be involved in loading of amino acids into the phloem. With the evolution of vascular plants, two vascular tissues were established, the xylem and the phloem. While the xylem functions in water and nutrient transport from the root to the shoot, phloem is important for long-distance transport of nutrients from source (e.g., mature leaves or roots) to sinks such as developing roots, flowers and seeds. In most herbaceous plants such as *Arabidopsis*, rice, or *Medicago*, phloem loading follows the apoplasmic route, where nutrients are loaded from the apoplast into the sieve element-companion cell complex of the collection phloem (Rennie and Turgeon, 2009). In addition, xylem to phloem amino acid transfer might occur along the transport pathway from source to sink (Pate et al., 1975, 1977). These loading steps into the collection or transport phloem require the activity of plasma membrane transporters.

In *Arabidopsis*, AtAAP3 function in the phloem seems to be restricted to the root (Okumoto et al., 2004), while AtAAP5 probably functions in import of amino acids into the companion cells (Brady et al., 2007; Zhang et al., 2008) of different organs including roots and leaves (Fischer et al., 1995; Cartwright et al., 2009; see also Tegeder and Rentsch, 2010). Preliminary results from the Tegeder lab indicate that AtAAP4 also plays a role in leaf phloem loading (Garneau and Tegeder, unpublished). AtAAP2 on the other hand is essential for phloem loading along the transport path (Hirner et al., 1998; Zhang et al., 2010). One rice and five *Medicago* proteins are related to the *Arabidopsis* phloem loaders and we cautiously speculate that they are involved in amino acid import into the sieve element/companion cells complex in legumes and monocot species. This prediction however requires proof through cellular and subcellular localization studies, and functional analysis in plants in

future. Nevertheless, it receives some support from the fact that other legume AAPs, specifically *Phaseolus vulgaris PvAAP1* and *Pisum sativum PsAAP1*, have also been localized to the phloem and group within cluster 3A (Tegeder et al., 2007; Tan et al., 2008).

Cluster 3B only contains seven rice proteins (OAAP11A–11G); eudicots are not represented. It is possible that this large group of monocot AAPs all function in phloem loading of amino acids in different tissues considering the similarity of subcluster 3B to 3A and the presence of only one rice AAP (and multiple eudicot sequences) in 3A. Note that the placement of OsAAP3 in cluster 3A only has weak phylogenetic support. Further, the lack of *Selaginella* and *Physcomitrella* sequences in cluster 3B suggests that AAPs developed independently in monocots, rather than the alternative, that AAPs of this group were lost in eudicots. None of the currently known eudicot amino acid transporters including AAPs from *Arabidopsis* (**Figure 3**), tomato (LeAAPs), potato (StAAPs), pea (PsAAPs), faba bean (VfAAPs), canola (BnAAPs), and *Ricinus* (RcAAPs) falls into cluster 3B (see Tan et al., 2008), providing further support for this hypothesis. Future research needs to determine if the monocot AAP proteins of cluster 3B differ in function from eudicot AAP proteins.

Amino acid permeases in cluster 4 are also divided into two subclusters. Cluster 4A holds three rice (OsAAP12A–12C) and two *Medicago* AAPs (MtAAP12A and 12B) with unknown function. Subcluster 4B contains *Arabidopsis* AtAAP6 related proteins and is branched into a group with AtAAP6 and two (duplicated) *Medicago* proteins, a related single rice protein (OsAAP6), and a group harboring *Arabidopsis* amino acid transporters AtAAP1 and AtAAP8. AtAAP6 is localized to the leaf xylem parenchyma (Okumoto et al., 2002). Although not directly involved in phloem loading, it is predicted to be important for xylem to phloem transfer of amino acids in *Arabidopsis* (Okumoto et al., 2002; Hunt et al., 2010), and the AAP6 relatives in legumes and monocots might have similar functions. It is interesting that one group within cluster 4B only contains two *Arabidopsis* transporters. It seems that *Arabidopsis* has gained two extra copies of AtAAP6: AtAAP1 and AtAAP8. Both AtAAP1 and AtAAP8 proteins are involved in seed loading, rather than phloem loading of amino acids (Schmidt et al., 2007; Sanders et al., 2009), supporting that they are AAP6 paralogs. It is tempting to hypothesize that monocot AAPs in group 4A function in seed loading as nothing is known to date concerning the function of OsAAP12A, B, or C.

Nevertheless, it is important to point out that *AAP* expression is generally not phloem or seed specific (see Ortiz-Lopez et al., 2000; Rentsch et al., 2007; Tegeder and Rentsch, 2010). For example, *Arabidopsis AAP*s (i.e., *AtAAP1* and *AtAAP5*) are also expressed in root epidermal and cortex cells suggesting that they fulfill additional functions in plants including amino acid uptake from the soil (Lee et al., 2007; Svennerstam et al., 2008; Cartwright et al., 2009). Further, the function of AAPs and other amino acid transporters including LHTs seems to be influenced by the physiology of the plant as nitrogen starvation and nitrate re-feeding affects their expression patterns (Liu and Bush, 2006).

In angiosperm, analysis of the relatedness of the rice and legume AAPs with *Arabidopsis* proteins might help with prediction of their function. For example a placement of rice and/or *Medicago* proteins with *Arabidopsis* AAP2, 3, 4, 5, and 6 might suggest a function in phloem loading. Interestingly, rice lacks close relatives of AAP2 and AAP4, and *Medicago* has no AAP3 and AAP5 phloem loaders. Some of the duplicated *Arabidopsis* AAPs may be functionally redundant. At least for AtAAP3 this appears to be the case, since mutant analysis did not result in a functional phenotype (Okumoto et al., 2004). On the other hand, some of the evolved AAPs in legumes (*Medicago*) and monocots seem not to be present in *Arabidopsis*/non-legume dicots (see **Figure 3**, subcluster 2B, 3B, and 4A) further supporting differences in AAP function among angiosperms. This is also in agreement with the large variation in the number of AAP proteins between *Arabidopsis* (8 proteins), *Medicago* (11 proteins), and rice (19 proteins). For example monocots might require additional or different amino acid transporters than eudicots due to differences in morphology and physiology between these distinct groups of seed plants. In legumes, additional AAP proteins might be needed for amino acid transport processes related to N_2 fixation and nodule function.

Taken together, AAPs are mainly found in euphyllophytes, including monocots and eudicots/legumes, which is in agreement with the main functions of AAPs in phloem and seed loading in support of amino acid translocation from source to sink (seeds). Non-vascular and non-seed vascular plants only have AAPs that are more closely related to AAP7, a transporter that remains to be characterized and might differ in function from the other AAPs.

LHTs EVOLVED PRIOR TO LAND PLANTS

Phylogenetic analysis revealed that LHTs are present in Charophytes (*Klebsormidium flaccidum*, 1 protein), non-vascular land plants (*P. patens*, 4 proteins), non-seed vascular plants (*S. moellendorffii*, 5 proteins), and seed plants (*A. thaliana*, 10 proteins; *O. sativa*, 6 proteins; *M. truncatula*, 13 proteins), demonstrating that LHTs evolved before the occurrence of early land plants (**Table 2**). The identified LHT proteins group into four clusters (**Table 2**; **Figure 4**). Cluster 1 includes LHT proteins of euphyllophytes, cluster 2 has five *Selaginella* and three *Physcomitrella* LHT proteins, cluster 3 contains euphyllophyte sequences and one *Physcomitrella* LHT and cluster 4 only contains one *Klebsormidium* protein.

Cluster 1 contains 8 of the 10 *Arabidopsis* AtLHTs, including AtLHT1, 2, 3, 5, 6, 8, 9, and 10. It appears that a duplication event has occurred in *Arabidopsis* and placement of the LHT proteins suggests that AtLHT2 and AtLHT5, AtLHT1 and AtLHT10, and AtLHT3 and AtLHT6, respectively are the result of such duplication. Relatives of AtLHTs are present in *Medicago* and 10 of the 13 MtLHTs are present in cluster 1. The presence of only four rice LHT sequences in group 1 indicates that LHTs were not as extensively duplicated in monocots as in eudicots. Recent studies have shown that *Arabidopsis* LHT transporters of cluster 1 including AtLHT1, 2, 4, 5, and 6 are expressed in male and female floral tissue, such as anther tissue, tapetum, mature pollen, pollen tubes, and pistil transmitting tissue (Hirner et al., 2006; Foster et al., 2008; see also Tegeder and Rentsch, 2010), and it was suggested that they might be essential for successful sexual plant reproduction. This is also in agreement with the observation that LHTs of cluster 1 are only present in flowering plant species. However, experimental proof for LHT function in reproduction is still missing, and AtLHTs of cluster 1 seem to have additional functions in plants as they are expressed in other organs besides flowers (Hirner

et al., 2006; see Arabidopsis eFP Browser, Winter et al., 2007). As for example recently demonstrated for AtLHT1, the transporter is important for amino acid uptake into root and mesophyll cells (Hirner et al., 2006; Svennerstam et al., 2008). LHT8 and LHT9 proteins of cluster 1 form separate subgroups. One group includes *Arabidopsis* AtLHT8 and an ortholog each in rice and *Medicago*, and the second group contains *Arabidopsis* AtLHT9, rice OsLHT9, and four closely related *Medicago* MtLHT9 transporters. Localization of these transporters has not been resolved and similar to most other LHTs, their physiological functions remain to be elucidated.

Cluster 2 includes three *P. patens* (PpLHT11A–C) and five *S. moellendorffii* LHT11 proteins (SmLHT11A–E) but none from Spermatophytes. *LHT11* genes may have evolved independently in *Physcomitrella* and *Selaginella* suggesting that in early land plants these LHTs serve functions in cellular amino acid transport processes that are not required in higher plants (see above). However, as in seed plants, Bryophytes and Lycophytes seem to need both LHTs and AAPs for growth and development (**Figures 1–4**).

Cluster 3 contains LHT4 and LHT7 proteins from angiosperms and *Physcomitrella* PpLHT4. Recent expression studies suggest that *Arabidopsis* AtLHT4 and AtLHT7 might be involved in reproduction, specifically in anther and pollen development (Bock et al., 2006; Foster et al., 2008). However, at least AtLHT4 has most certainly additional functions since it is also expressed in root and stem (Winter et al., 2007). This might explain its phylogenetic divergence from other LHTs and its placement with PpLHT4. In early land plants, transporters may be critical for amino acid movement over relatively short distances. As plants colonized dry land, translocation of amino acids from source to sink cells occurred probably by cell to cell transport (symplasmic) and between cells (apoplasmic), especially in non-vascular mosses (Trachtenberg and Zamski, 1978; Reinhart and Thomas, 1981). Uptake of the apoplasmic amino acids required membrane proteins including H^+-coupled, high affinity LHT symporters, as indicated by the phylogenetic analysis.

Cluster 4 only contains a LHT protein from green algae *Klebsormidium* called KfLHT13, suggesting its evolutionary divergence from LHTs of land plants and differences in function. While we are not aware of amino acid transport studies in Charophytes, research with Chlorophytes such a *Chlamydomonas* and *Chlorella* spp. and marine microalgae demonstrate that in algae different transport systems are present (Kirk and Kirk, 1978a; Cho et al., 1981; Cho and Komor, 1985; Shehawy and Kleiner, 2001; Kato et al., 2006; see also Flynn and Butler, 1986 and references within). Although an LHT transporter was only found in *Klebsormidium*, the screened charophyte sequences were obtained from EST projects and we predict that LHTs are also present in other charophytes besides *Klebsormidium*. Placement of KfLHT13 further supports that LHTs have evolved before land plants and that their function is important to green algae as well.

Gene function of lysine–histidine-like transporters was likely important in ancestors of plants, as a gene is detected in a charophyte, contributing to its high affinity and substrate selectivity for neutral and acid amino acids. Localization and expression studies of *Arabidopsis AtLHTs* suggest that, in addition to other functions, LHTs have a major role in sexual plant reproduction in seed plants. This also indicates a difference in LHT function between angiosperms and non-seed/non-land plants. While LHT functions still need to be demonstrated *in planta*, this is in agreement with the phylogenetic analysis showing a grouping of angiosperm LHTs while LHT proteins from non-vascular and non-seed plants, and green algae are present in separate groups.

Based on the phylogeny, genes in the LHT family of land plants likely arose from an ancestral gene similar to the charophyte LHT. The ancestral gene diversified as plants colonized dry land, as seen by the presence of multiple LHT in moss, a non-vascular plant. In contrast, no algal genes encoding AAP transporters were detected in our analysis of the Charophytes, but it may be too early to conclude whether genes belonging to the AAP family are present, as the charophyte genome has not been sequenced completely. Algae generally acquire amino acids from the environment for growth, and some variation with respect of the kind and amount of amino acids that are taken up has been observed between and within species (Cho et al., 1981; Cho and Komor, 1983, 1985; Flynn and Butler, 1986; Kato et al., 2006). In addition, leakage of amino acids from the cells into the apoplast might occur and requires transporters for retrieval. Physiological studies have demonstrated that in algae active transport systems with varying specificity and affinities (high and low) are present (Kirk and Kirk, 1978a,b; Sauer et al., 1983; Cho and Komor, 1985; Shehawy and Kleiner, 2001; Kato et al., 2006; see also Flynn and Butler, 1986), which might point to the presence of both, LHT and AAP transporters, or additional amino acid transporters in Charophyte algae.

CONCLUSION

Recent functional studies support that AAP and LHT proteins have essential roles in transport of a broad range of amino acids in eudicots (see Tegeder and Rentsch, 2010). Here, phylogenetic analysis supports that AAPs, which generally present moderate and low affinity systems for neutral and acidic amino acids, are important to land plants with a main function in phloem loading and that they are not required in red algae or green algae (Charophytes or Chlorophytes). In contrast, LHTs are found in green algae, non-seed plants and angiosperms suggesting the need for high affinity amino acid transporters across the different organisms.

Both AAPs and LHTs were found in all land plants analyzed consistent with essential and distinct functions for both transporter families. To date, information on the role of AAP and LHT transporters is almost exclusively based on studies in *Arabidopsis* and in some cases in legumes, and suggests differences between AAPs and LHTs in substrate selectivity, transport affinity and cellular function (see Rentsch et al., 2007; Tegeder and Rentsch, 2010; see also above). However, phylogenetic analyses indicates that function of some AAP and LHT transporters diverged in monocots, non-seed vascular plants, non-vascular plants and green algae, and future studies need to address the role of the amino acid transporters across land plants and in green algae.

MATERIALS AND METHODS

IDENTIFICATION AND ANNOTATION OF AAP AND LHT PROTEINS

Genome sequences are available for *Arabidopsis thaliana*, rice (*Oryza sativa*), *Medicago truncatula*, *Selaginella moellendorffii*, *Physcomitrella patens*, *Chlamydomonas reinhardtii*, and *Volvox carterii*. AAP and LHT sequences were selected from rice, *M.*

truncatula, *S. moellendorffii*, and *P. patens* predicted protein sequences using BLAST searches with known *Arabidopsis* AAP and LHT transporters (see **Tables 1** and **2**) on the Phytozome website[2]. The same database was searched for AAP and LHT protein sequences from the Chlorophytes *C. reinhardtii* and *V. carterii*. Dr. Charles F. Delwiche and Dr. James Thierer, University of Maryland provided support by screening their EST (Expressed Sequence Tag) databases for AAP and LHT relatives in charophytes, specifically in *Penium marinum*, *Spirogyra praetensis*, *Coleochaete* sp., and *Chaetosphaeridium globosum*, *Mesostigma viride*, *Nitella hyalina*, *Klebsormidium flaccidum*, *Chlorokybus atmosphyticus*[3]. In addition, the genome (protein) sequences of the red algae *Galdieria sulfuraria* available through http://genomics.msu.edu/cgi-bin/galdieria/blast.cgi (Barbier et al., 2005) and *Cyanidioschyzon merolae* at http://merolae.biol.s.u-tokyo.ac.jp/and were searched for the presence of AAPs and LHTs.

SEQUENCE ALIGNMENTS AND PHYLOGENETIC ANALYSIS

Multiple protein sequence alignments were generated with Clustal X (Thompson et al., 2007) and, for comparison, with MUSCLE (Edgar, 2004). Phylogenetic analysis was performed through the iPlant Collaborative website[4]. Maximum-likelihood analysis was done using PhyML 3.0 (Guindon and Gascuel, 2003; Guindon et al., 2010) and statistical analysis of phylogenetic trees was performed using a Shimodaira–Hasegawa-like test (SH-like test; Shimodaira and Hasegawa, 1999; Buckley et al., 2001). The values for SH-like branch support are presented at the nodes on the trees. Values above 0.9 show significant phylogenetic support. Trees were visualized using the FigTree program[5]

NAMING OF AAP AND LHT TRANSPORTERS

The identified AAP and LHT sequences were named based on clustering with *Arabidopsis* AtAAP and AtLHT protein sequences. In *Arabidopsis* 8 AAP (AtAAP1–8) and 10 LHT (AtLHT1–10) transporters have been previously identified (see Rentsch et al., 2007). Phylogenetic grouping was used to name the transporters from other species. In cases where more than one *Arabidopsis* relative was found from a given species, letter labeling was chosen in addition. For example, three rice relatives of AtAAP7 were named OsAAP7A, 7B, and 7C. Transporters that did not group with *Arabidopsis* proteins were given numbers not found for the *Arabidopsis* transporters such as AAP9 or LHT11.

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[2]http://phytozome.net
[3]http://www.clfs.umd.edu/labs/delwiche/Charophyte.html
[4]http://www.iplantcollaborative.org/
[5]http://tree.bio.ed.ac.uk/software/figtree/.

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